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The human circulating miRNome reflects multiple organ disease risks in association with short-term exposure to traffic-related air pollution

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Abstract

Traffic-related air pollution is a complex mixture of particulate matter (PM) and gaseous pollutants, such as nitrogen dioxide (NO₂). PM exposure contributes to the pathogenesis of many diseases including several types of cancer, as well as pulmonary, cardiovascular and neurodegenerative diseases. Also exposure to NO₂ has been related to increased cardiovascular mortality. In search of an early diagnostic biomarker for improved air pollution-associated health risk assessment, recent human studies have shown that certain circulating miRNAs are altered upon exposure to traffic-related air pollutants. Here, we present for the first time a global analysis of the circulating miRNA genome in an experimental cross-over study of a human population exposed to traffic-related air pollution. By utilizing next-generation sequencing technology and detailed real-time exposure measurements we identified 54 circulating miRNAs to be dose- and pollutant species-dependently associated with PM₁₀, PM_{2.5}, black carbon, ultrafine particles and NO₂ already after 2 hours of exposure. Bioinformatics analysis suggests that these circulating miRNAs actually reflect the adverse consequences of traffic pollution-induced toxicity in target tissues including the lung, heart, kidney and brain. This study shows the strong potential of circulating miRNAs as novel biomarkers for environmental health risk assessment.

Keywords: air pollution; diesel exhaust; extracellular microRNAs; biomarkers; health risk assessment; liquid biopsies

Introduction

Numerous epidemiological studies have associated exposure to traffic-related air pollution (TRAP) with increased risk of cardiovascular disease^{1,2}, respiratory disease³, several types of cancer, including lung and breast cancer^{4,5}, and more recently also of neurodegenerative diseases^{6,7} and kidney disease⁸. A number of gaseous pollutants such as nitrogen dioxide (NO₂) as well as particulate matter components (PM) are routinely monitored to characterize TRAP exposure. PM is a complex mixture of fine particles with a diameter of 10 µm or less (PM₁₀), a diameter of 2.5 µm or less (PM_{2.5}), black carbon (BC), ultrafine particles with a diameter of 0.1 µm or less (UFP) and soot⁹. Upon inhalation PM penetrates deeply into the lungs from where, depending on their size, particles are capable of entering the circulation and being distributed to distal organs such as the heart, spleen or liver⁹⁻¹¹. It has even been reported that ultrafine PM crosses the blood-brain barrier and translocates from the circulation to the brain¹². Further, it has been demonstrated that PM triggers oxidative stress in the respiratory tract and that this might induce a systemic inflammatory cascade, thus increasing the risk for respiratory and cardiovascular diseases¹³. Presumably, pollutants, once distributed over the whole body, may cause a similar cascade of oxidative stress and inflammation in target organs, thereby increasing risks for cancer and neurodegenerative disease¹⁴. However, the precise molecular mechanisms that link TRAP exposure to increased disease risks are still poorly understood which hampers the development of dedicated biomarkers capable of informing on relevant molecular mechanisms of action.

Several studies have thus highlighted the impact of environmental exposure on gene expression profiles¹⁵, DNA-methylation patterns¹⁶, and p53 status¹⁷. More recently, environmental

exposure-induced alterations in microRNA (miRNA) levels have been described¹⁸. These small non-coding RNAs are involved in the posttranscriptional regulation of gene expression, and consequently are involved in virtually all cellular processes¹⁹. Furthermore, while these fine-tuners of gene expression are capable of adjusting to internal and external conditions, they also exhibit tissue/organ specific expression patterns²⁰. As a consequence of organ injury, cells may leak their content including the highly stable protein-bound miRNAs, into the peripheral circulation²¹. Given the fact that certain miRNAs are more abundantly expressed in specific organs, circulating miRNA (cmiRNA) signatures may thus also reflect organ-specific responses to exposure²². Furthermore, through active secretion, extracellular vesicle-bound cmiRNAs may act as mediators in intercellular and interorgan communication²³. Therefore, cmiRNAs leaked or released from organs into the circulation, have become a new promising class of biomarkers capable of non-invasively interrogating organ pathogenesis and organ-toxic mechanisms from so called 'liquid biopsies'²².

To date, most reported air pollution-induced changes in miRNA expression have been identified in solid tissues in animal models²⁴. The first evidence on PM exposure-related modifications in cmiRNA levels in humans was provided through investigating healthy steel plant workers. This study identified 2 vesicle-associated miRNAs that were elevated after occupational exposure to metal-rich PM¹³. Additionally, a study on long term exposure to ambient air pollution (6 month or 1 year) identified the elevation of 5 vesicle-associated cmiRNAs in the serum of healthy subjects²⁵. Another study among children identified 2 cmiRNAs in the extracellular fraction of saliva to be significantly altered with long-term ultrafine PM exposure²⁶.

These studies provided evidence that the extracellular miRNA genome (miRNome) is affected by TRAP exposure through utilizing targeted approaches, and were consequently restricted to

analyzing *a priori* known air pollution-associated miRNAs. In the current study, we present for the first time a global analysis of the circulating miRNome by applying next generation sequencing technology and real-time exposure measurements in an experimental cross-over study of human volunteers (n=24) following short-term traffic-related air pollution exposure. This study demonstrates the potential of circulating miRNAs as novel biomarkers for health risk assessment in relation to environmental exposure-induced target tissue pathogenesis.

Methods

Selection of the population

Plasma samples were collected during a randomized experimental crossover study in which non-smoking participants, either healthy or suffering from ischemic heart disease (IHD) or chronic obstructive pulmonary disease (COPD), walked for 2 hours along Oxford Street in London (where only diesel-powered buses and taxicabs are permitted). In a separate session the same subjects also walked for 2 hours through traffic-free Hyde Park. In order to balance between sufficient exposure and what is acceptable for the participants we decided for a 2 hours exposure window which also has been applied successfully in an earlier study²⁷. Sessions were segregated by a period of more than 3 weeks. Participants walked about 6 km during each exposure period, at a steady pace on predefined paths, resting for 15 minutes every half hour. During each exposure session, personal ambient air pollution levels of PM were assessed using a real-time condensation particle counter (Model 3007, TSI). Particles smaller than 2.5 µm in aerodynamic diameter were collected on an air sampler. BC was measured according to the National Institute for Occupational Safety and Health guidelines (Method 5040, Sunset Laboratory). Using a sampling pump, NO₂ was collected on C18 Sep-Pak cartridges coated with potassium hydroxide

and triethanolamine and analyzed using ion chromatography²⁷. Our comprehensive monitoring design aimed to account for each of the major ambient air pollution factors that differed between sites. To the best of our knowledge there are no major differences with respect to any other factors not taken into account. In total 119 subjects completed the exposure assessment²⁸. For the present study, we selected 24 non-smoking participants, based on the maximal change in PM exposure between Hyde Park and Oxford Street while balancing for gender (Table 1). The study was approved by the UK National Research Ethics Service (London City Road and Hampstead Ethics Committee; Research Ethics Number 12/LO/1064, 01/08/2012). All participants gave written informed consent. The studies were conducted in accordance with the approved guidelines and regulations.

	# Subjects	Age, mean (SD)	BMI, mean (SD)	# Healthy	# COPD	# IHD
All subjects	24	65.1 (7.7)	25 (4.8)	8	8	8
Male	12	65(10)	24.9(3.5)	3	4	5
Female	12	64.9(5.1)	25.1(6)	5	4	3

Table 1: Study population data.

Analytical procedures

After each exposure session the participants were transferred to the Royal Brompton Hospital where blood samples were collected. Plasma samples were recovered from plasma separator tubes following centrifugation of whole blood at 1,600g for 10 min at 4°C. To avoid

contamination of the plasma cmiRNAs with blood cell-derived miRNAs, the plasma was subjected to a second centrifugation step at 16,000g before being stored at -80°C²⁹. Plasma cmiRNAs were isolated using the miRNeasy Serum/Plasma kit (Qiagen) and the quality was evaluated on a Bioanalyzer using the small RNA Kit (Agilent). Samples were included in the analysis when showing a minimum of 1 ng RNA yield and a peak at 21 nucleotides. The sequencing libraries were prepared using the TruSeq Small RNA-Seq Preparation Kit (Illumina), and sequenced by a HiSeq 2500 (Illumina)³⁰ according to the manufacture's protocol (GEO accession: GSE106221).

Statistical data analysis

The quality of the sequencing data was assessed by FastQC and subsequently processed using the miRge pipeline and miRBase (release 21)^{31,32}. In R, we set a threshold for a cmiRNA to be detected with a minimum of 100 raw sequencing reads across all samples. The raw data were quantile normalized and transformed to log2 counts per million (logCPM) using the 'voom' function of the package 'limma'³³. Technical confounders (inter-day variations in library preparation and isolation batch effects) were adjusted for by applying a linear mixed model approach using the R package 'lme4'³⁴. After de-noising the data we used a multivariate normal model to find associations between the air pollutant exposure and cmiRNA using the R package 'nlme'³⁵. For each exposure, an independent analysis was performed thereby also adjusting for sex, age, BMI and disease state (healthy, IHD or COPD). Walks were carried out Monday to Thursday. No attempt was made to match the day of the week when subjects returned for the second session as this would have been too restrictive to the subjects. However, the 'traffic exposure' site (Oxford Street) was only open to buses and taxis, with diesel buses representing

the dominant emission source. As bus flows were consistent across all study days, day of the week was not regarded a significant confounder. Resulting p-values were controlled by the False Discovery Rate (FDR) at 5%³⁶.

Potential gene targets were derived from the experimentally validated miRNA-target interactions database miRTarBase (release 6) by only considering strong miRNA-gene interactions that have been validated by reporter assay, western blot or qPCR. Overrepresentation analysis of identified KEGG pathways retrieved from the potential gene targets was performed in ConsensusPathDB (release 32), using the gene targets for all detected cmiRNAs as a background list. Connections between pathways and diseases were retrieved from the KEGG DISEASE Database (updated: September 16, 2016).

To identify potential organs of origin of the detected cmiRNAs, we examined miRNA tissue enrichment using publicly available small RNA-seq data sets. The data were obtained from the Human miRNA Expression Database³⁷ as well as from the sequence read archive (SRA012516)³⁸. The final dataset comprised 101 small RNA sequencing samples across 8 organs known to accumulate inhaled fine PM⁹⁻¹¹. We applied a tissue specificity index (TSI), as described originally for mRNAs by Yanai et al., for each of the significant air pollutant exposure-associated cmiRNAs. The index is defined as:

$$TSI = \frac{\sum_i^N (1 - x_i)}{N - 1}$$

Where N is the number of organs included in the analysis and x_i is the expression of a miRNA, normalized by the value of the highest expressed miRNA³⁹. A $TSI < 0.5$ indicates that a miRNA

is expressed across all the tissues whereas a higher TSI indicates that a miRNA is enriched in one or a few tissues.

Results

Exposure range

The subjects analyzed for cmiRNAs were exposed to a mean ambient air NO₂ level of 7.9 (CI 5.9-9.8) µg/m³ in Hyde Park and 18.1 (CI 15.1-21.1) µg/m³ in Oxford Street. For PM_{2.5} the mean exposure level in Hyde Park was 5.6 (CI 4.5-6.8) µg/m³ and 25.6 (CI 21-30.2) µg/m³ in Oxford Street. For BC the exposure level was 1.0 (CI 0.8-1.3) µg/m³ in Hyde Park and 11.4 (CI 9.9-12.8) µg/m³ in Oxford Street and for PM₁₀ 16.0 (CI 12.5-19.5) µg/m³ in Hyde Park and 37.0 (CI 32.2-41.7) µg/m³ in Oxford Street. For UFP the mean particle count was 5,975 (CI 4815.4-7133.8) thousands/cm³ in Hyde Park and 28,656 (CI 25803-31509) thousands/cm³ in Oxford Street. The exposure level densities for UFP, PM_{2.5}, BC, PM₁₀ and NO₂ are presented in Figure 1.

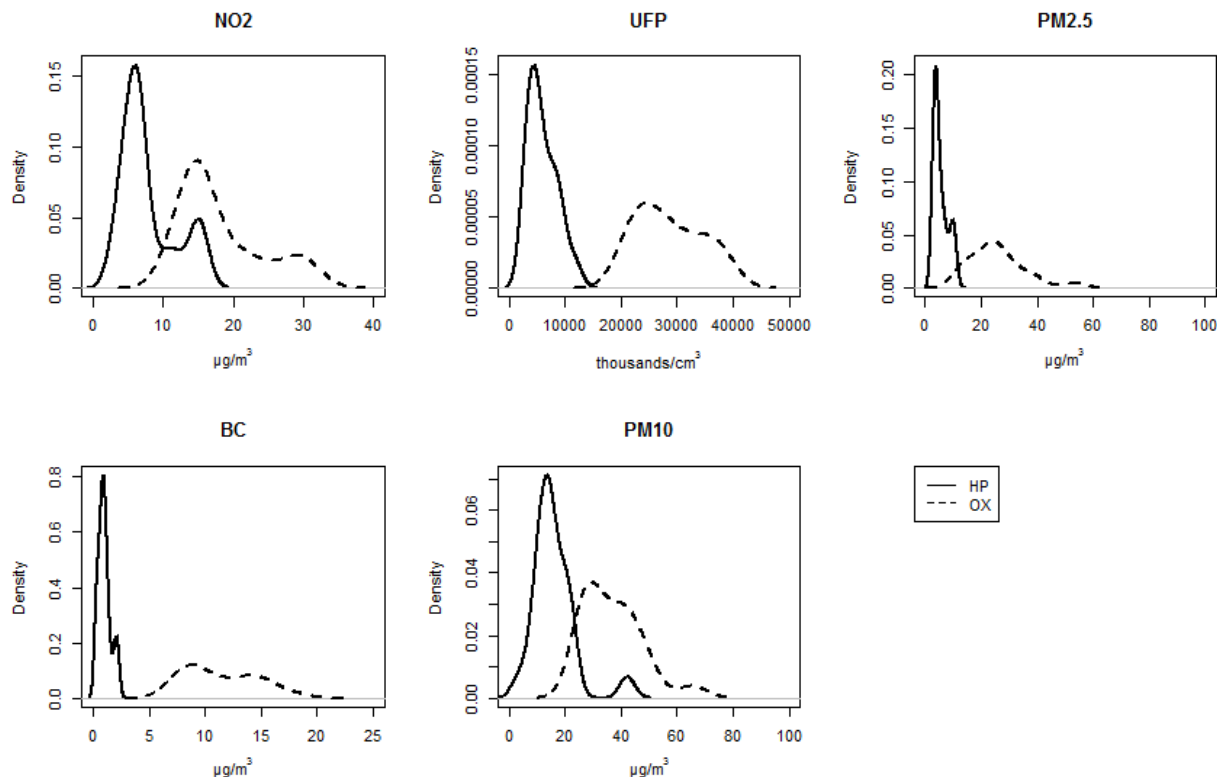


Figure 1. TRAP density of the included subjects for cmiRNA analysis. The figure shows the exposure density of NO₂, UFP, PM_{2.5}, BC and PM₁₀ for the included subjects in Hyde Park (HP) and Oxford Street (OX).

Small RNA-sequencing

The sequencing of the 48 samples from 24 subjects yielded a mean of 5.6 million (+/- 2.8 million) high quality, preprocessed reads per sample of which on average 3.8 million (+/- 2.1 million) could be assigned to the miRNA sequences from miRBase (release 21). Principal component analysis revealed that 2 outliers were present in the data. As a consequence these 2 subjects, as well as 2 further subjects with missing exposure measurements, were excluded from the analysis. The excluded subjects were not separated by any specific characteristics used in the analysis (Supplementary Table S1). In total, we identified 595 cmiRNAs across all plasma samples. The most abundant plasma cmiRNA was miR-486-5p with 19.4 logCPM, followed by miR-92a (16.6 logCPM), and miR-22-3p (14.4 logCPM). The upper quantile of all identified

cmiRNAs ranged from 4.5 logCPM to 19.4 logCPM, whereas the lower quantile ranged from -3.5 to -1.4 logCPM. The majority of the cmiRNAs present in the plasma varied between 4.5 and -3.5 logCPM.

TRAP-associated cmiRNAs

Using the multivariate normal model in combination with the linear mixed model for correction of technical random effects, we identified cmiRNAs significantly associated with pollutant-specific exposure levels (Figure 2). We observed 6 cmiRNAs to be significantly associated with the NO₂ levels, of which 4 were negatively and 2 positively correlated with exposure. Further, we identified 7 cmiRNAs, 5 negatively and 2 positively, associated with UFP counts. Analysis of PM_{2.5} levels resulted in 23 cmiRNAs, of which 12 were negatively and 11 positively associated with PM_{2.5} levels. We observed 26 cmiRNAs to be associated with BC levels of which 14 were negatively and 12 positively associated with exposure. For PM₁₀ levels we identified 28 cmiRNAs of which 17 were negatively and 11 positively associated with PM₁₀ levels. Together, the traffic-related air pollutants (NO₂, UFP, PM_{2.5}, BC and PM₁₀) were associated to 54 unique cmiRNAs (Supplementary Table S2). Figure 3 presents the different plasma levels of those 9 cmiRNAs that were declared significant based on the more stringent Bonferroni correction (ensuring a family-wise error rate <0.05). When comparing the read outs after exposure in Hyde Park and upon exposure in Oxford Street, we observed decreased miR-133a-3p levels in 95% of the subjects, in 85 % decreased miR-193b-3p levels, in 80% increased miR-1224-5p levels, in 85% decreased miR-433-3p levels, in 80% decreased miR-145-5p levels, in 65% decreased miR-27a-5p levels, in 60% decreased miR-580-3p levels, in 55% increased miR-3127-5p levels and in 75% decreased miR-6716-3p levels. This pair-wise comparison clearly demonstrates the consistency in cmiRNA response to ambient air pollutant exposure among the subjects. We did

not detect any significant results upon stratification by disease state (COPD or IHD vs Healthy)
or gender due to the limited number of samples.

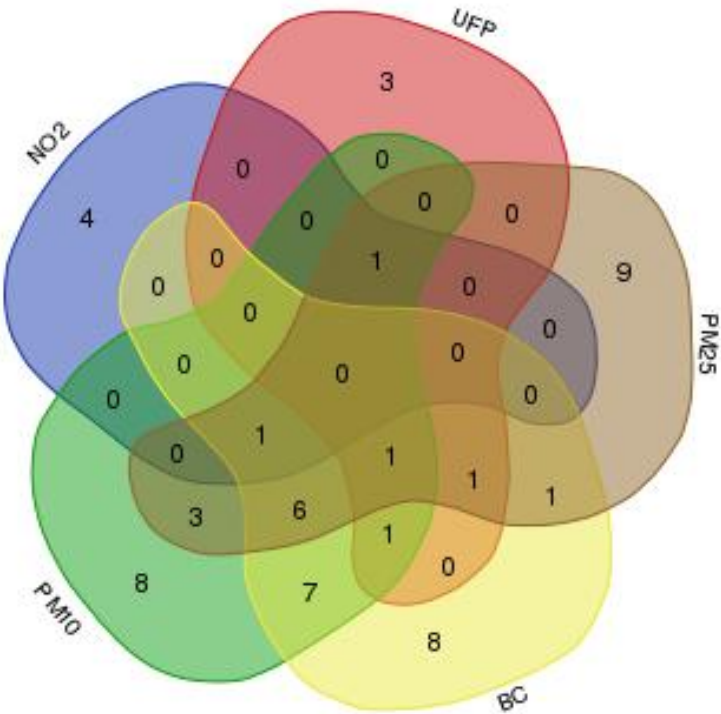


Figure 2. Pollutant-specific cmiRNAs associated with TRAP exposure. The figure shows the overlap as well as the specificity of the pollutant-specific cmiRNAs associated with exposure to NO₂, UFP, PM_{2.5}, BC and PM₁₀ of the included subjects in Hyde Park and Oxford Street.

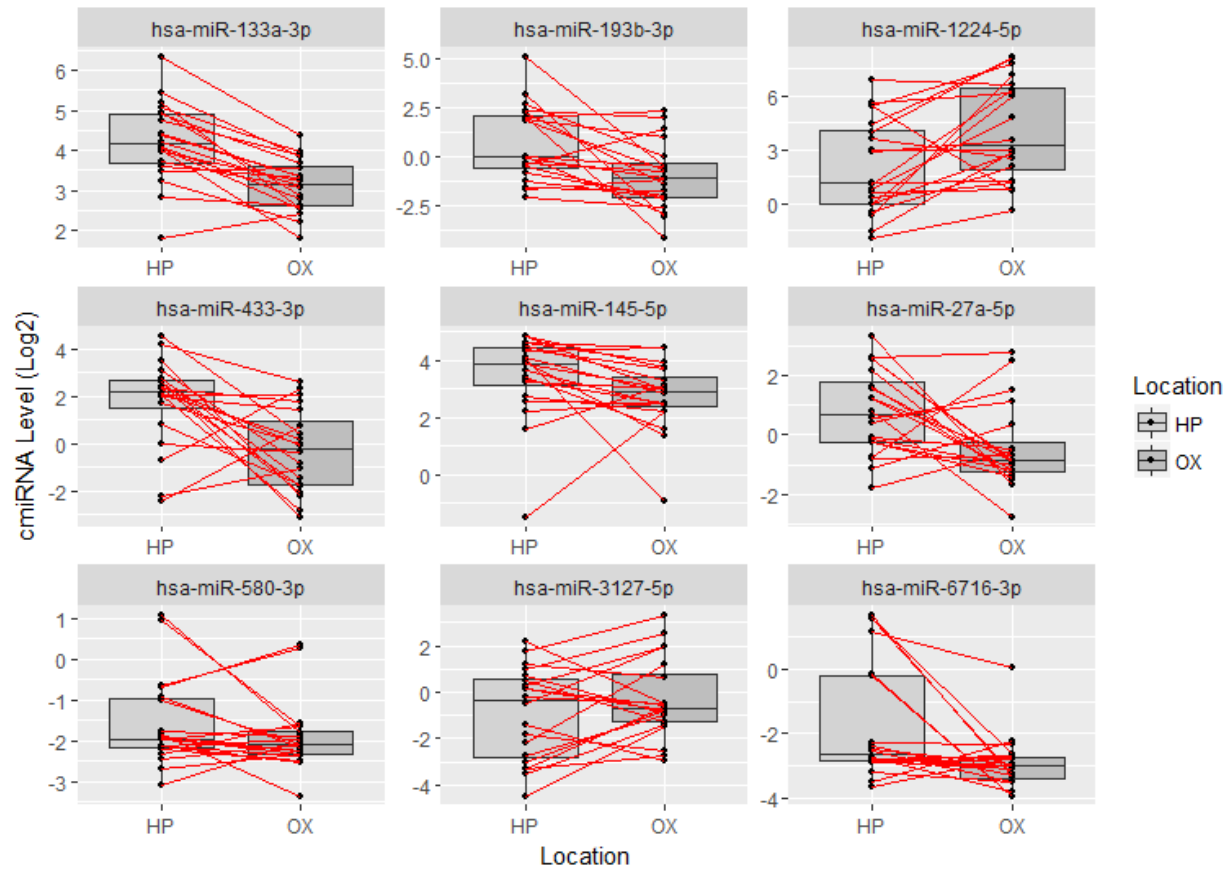


Figure 3. Top 9 significant cmiRNAs affected by TRAP exposure. The figure shows the plasma levels of the top 9 significant TRAP-associated cmiRNAs for each subject after walking in Hyde Park (HP) and along Oxford Street (OX). The red line connects the two measurements per subject.

Origin of circulating miRNAs

To assess the tissue origin of the detected cmiRNAs, we examined miRNA tissue enrichment using publicly available small RNA-seq data sets. From the 54 TRAP-affected cmiRNAs, we identified 7 to be highly expressed ($\log_2\text{CPM} > 10$) in the lung, 8 in the breast, 3 in the heart, 6 in the kidney, 10 in the liver, 8 in the pancreas, 7 in the spleen and 7 in the brain.

Of the cmiRNAs significantly associated with air pollutant exposure, only 1 (miR-193b-3p) overlapped with the list of miRNAs associated with hemolysis, indicating that the potential plasma contamination with miRNAs from red blood cells was minimal⁴⁰.

By applying a tissue specificity index (TSI) we found that the cmiRNAs that were most abundant in the plasma, notably miR-25-3p, miR-30d-5p, let-7i-5p, miR-107 (plasma level > 5 log₂CPM), showed a low TSI and were consequently highly abundantly expressed across all target tissues. By contrast, low abundant cmiRNAs (plasma level < 5 logCPM) showed a high TSI and appeared to be enriched in only one or just a few tissues. The miRNAs miR-133a-3p and miR-499a-5p, both enriched in the heart with a TSI of 0.60 and 0.64, were present in the plasma at a mean of 3.7 and -2.1 logCPM, respectively. The brain-enriched miR-433-3p showed a TSI of 0.58 with a plasma level of 0.7 logCPM, while the brain- and pancreas-enriched miR-1224-5p showed a TSI of 0.68 and was detected in the plasma at 3 logCPM. We identified the kidney-enriched miRNAs miR-3678-3p, miR-6741-3p, miR-6865-5p, miR-4687-5p, hsa-miR-627-3p and miR-636, all with a TSI > 0.68 and plasma levels < 3.8 logCPM. Furthermore, we identified miR-6514-3p to be enriched in the breast, with a TSI of 1 and a plasma level of -2.9 logCPM (Figure 4).

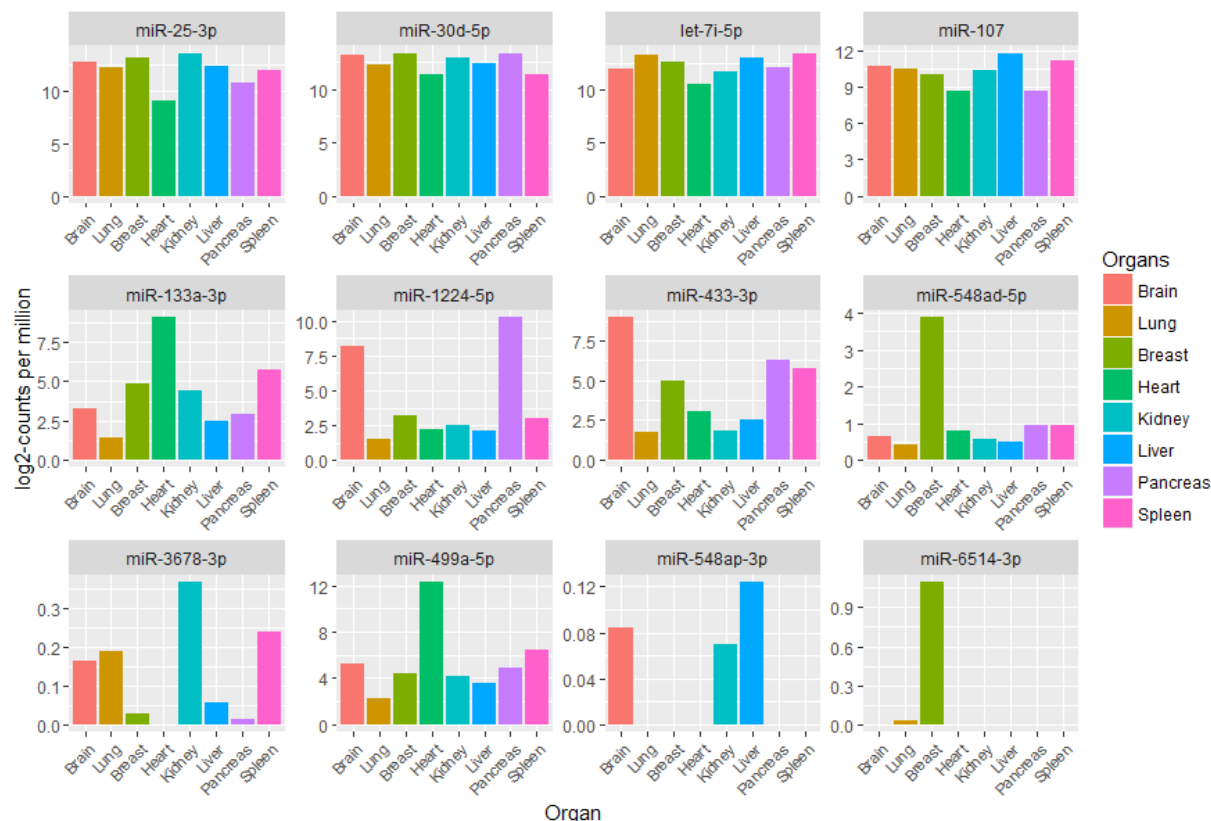


Figure 4. Expression of TRAP-associated cmRNAs across organs known to be targeted by inhaled air

pollutants. The figure shows the mean expression of the high and low abundant cmRNAs across organs associated with air pollutant accumulation in logCPM. The data were obtained from the Human miRNA Expression Database³⁷ as well as from the sequence read archive (SRA012516)³⁸. The high abundant cmRNAs appeared to be housekeeping miRNAs (miR-25-3p, miR-30d-5p, let-7i-5p, and miR-107), while the low abundant cmRNAs seemed to show organ specificity/enrichment.

Disease associations, Target genes and Pathway analysis

To investigate the potential involvement of the 54 identified cmRNAs in air pollutant-related diseases, we explored known disease-miRNA interactions from published studies found in PubMed and miRWalk (version 2.0). We identified 8 interactions with breast cancer, 3 interactions with cardiovascular diseases, 5 interactions with inflammation, 8 interactions with respiratory disease (incl. lung cancer and Asthma), 6 interactions with neurodegenerative

260 diseases (incl. Alzheimer's Disease and Parkinson) and 4 interaction with kidney diseases (Table
 261 2).

Disease	# Associations/interactions	miRNAs	References
Breast cancer	8	miR-107↑; miR-125a-5p↓; miR-145-5p↓; miR-150-5p↓; miR-193b-3p↓; miR-342-3p↓; miR-34a-5p↓; miR-98-5p↑	41-48
Cardiovascular Disease	3	miR-133a↓; miR-145-5p↓; miR-499a-5p↓	49-51
Inflammation	5	let-7i-5p↑; miR-107↑; miR-223-3p↓; miR-25-3p↑; miR-miR-98-5p↑	52-55
Kidney Diseases	4	miR-25-3p↑; miR-148a-3p↑; miR-150-5p↓; miR-223-3p↓	56
Neurodegenerative diseases (incl. Alzheimer's Disease and Parkinson)	6	miR-107↑; miR-1224-5p↑; miR-145-5p↓; miR-34a-5p↓; miR-433-3p↓; miR-885-5p↓	54,57-61
Respiratory Diseases (incl. lung	8	let-7i-5p↑; miR-107↑;	43,52,62,63

cancer and asthma)		miR-125a-5p↓; miR-145-5p ↓; miR-148a-3p↑; miR-223-3p ↓; miR-574-5p ↑ miR-98-5p ↑	
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Table 2. Overlap between miRNAs found to be altered in diseased tissue and cmiRNAs to be associated with air pollutant exposure. (↑) indicates cmiRNAs to be positively associated and (↓) negatively associated with the exposure levels. The miRNAs marked in bold show the same directionality in subjects exposed air pollution exposure and in patients suffering from a particular disease.

To further describe the functional relevance of air pollutant exposure-associated cmiRNAs, we explored their potential interactions with gene targets from the experimentally validated miRNA-target interactions database miRTarBase (release 6, only strong interactions). We identified a set of 491 target genes for the air pollutant exposure-associated 54 cmiRNAs. We explored the relationship between these cmiRNAs and their respective target genes by performing an overrepresentation analysis of KEGG pathways using ConsensusPathDB (release 32). This analysis identified 31 KEGG pathways (Supplementary Table S3). Notably, the list of overrepresented pathways includes significantly modified cancer-related pathways such as microRNAs in cancer, specifically breast cancer, non-small cell lung cancer and small cell lung cancer as well as signaling pathways such as the PI3K-Akt signaling pathway and the p53 signaling pathway.

Discussion

In this study, we evaluated the global circulating miRNome in plasma from human subjects exposed to ambient TRAP for only 2 hours by using NGS. We identified 54 cmiRNAs that appear to be involved in the molecular response to NO₂, UFP, PM_{2.5}, BC and PM₁₀ exposure. Next, we gathered information on tissue-specific miRNAs from those organs known to be targeted by ambient air pollutants. We found that the most abundant cmiRNAs present in plasma are equally expressed in all organs known to be targeted by TRAP. By contrast, the cmiRNAs present at low plasma levels appeared to be specifically enriched in certain target organs (TSI >0.5) (Figure 4). The inverse association between high TSI and lower plasma level (correlation coefficient = -0.68) thus suggests that lower expressed (logCPM < 5) cmiRNAs might particularly represent tissue-specific biomarkers. Therefore, we hypothesize that the low abundant cmiRNAs are promising markers for the evaluation of tissue and organ conditions reflected by non-invasive ‘liquid biopsies’. In contrast to studies in which miRNAs may leak into the circulation upon high exposures to toxic compounds, such as a consequence of acetaminophen-induced cytotoxicity in the liver³⁰, ambient TRAP levels are unlikely to induce cell death within 2 hours of time, implying that the induced alterations of cmiRNAs are more likely to reflect the active release of specific miRNAs into the circulation¹³.

Ambient TRAP exposure has been implicated in the development of cardiovascular disease¹, respiratory disease³, kidney disease⁸, several types of cancer^{4,5}, as well as in neurodegenerative diseases^{6,7}. This is now also suggested on the molecular level by our results on tissue- and disease-specific cmiRNAs following short-term exposure to ambient TRAP. We demonstrated that the heart-enriched miR-133a-3p was significantly decreased in the plasma of 95 percent of the participants upon increased exposure. Downregulation or deletion of this miRNA has been associated with cardiac hypertrophy, severe fibrosis and heart failure. A study on the partial

knockdown of miR-133 in mice has shown to induce hypertrophy during cardiac diseases⁶⁴. Furthermore, the complete knockout of both miR-133 genes resulted in late embryonic or neonatal lethality due to ventricular septal defects in the majority of the mice, whereas mice that survived to adulthood developed extensive myocardial fibrosis and finally died from heart failure⁶⁵.

The smooth muscle cell-enriched miR-145-5p was significantly decreased in 80% of the participants upon exposure to TRAP. This miRNA, which has been reported to be downregulated in the carotid artery upon mechanical injury, is involved in regulation of smooth muscle contractility and the stress response to vessel injury, and thus is involved in cardiovascular diseases⁵⁰.

Furthermore, we observed TRAP-dependent downregulation of the heart-enriched miR-499a-5p, a miRNA that is known to be downregulated under pathological heart conditions and has been suggested as a pharmacological target in the treatment of apoptosis-related cardiac disease, including myocardial infarction⁵¹.

Overall, the two heart-enriched miRNAs miR-133a-3p and miR-499a-5p as well as the smooth muscle cell-enriched miR-145-5p play a crucial role in the development of cardiovascular diseases which is known to be associated with exposure to TRAP, and therefore add potential value to a miRNA-based biomarker catalogue for cardiovascular disease prevention and biomarker-based environmental health risk assessment.

We observed, in 85% of the subjects decreased plasma levels of miR-193b-3p in relation to higher TRAP exposure levels. The downregulation of this miRNA in breast tissue is known to enhance tumor progression and invasion in human breast cancer⁴⁵. Also, the plasma level of

miR-145-5p was decreased in 80 % of participants, a miRNA that inhibits growth and migration of breast cancer⁶⁶. Furthermore, miR-145-5p has also been identified to inhibit the proliferation of non-small cell lung cancer cells by targeting the oncogene *c-Myc*, and increasing the expression of miR-145 has been suggested as a novel approach for the treatment of lung cancer⁴³.

Potentially, the TRAP-induced inhibitions of miR-193b-3p and miR-145-5p play a role in the complex progress of cancer development upon ambient air pollution exposure. Furthermore, in association with air pollutant exposure levels we identified another 4 decreased cmiRNAs (miR-125a-5, miR-150-5p, miR-342-3p and miR-34a-5p) and 2 increased cmiRNAs (miR-107 and miR-98-5p) for which a role in breast and/or lung cancer development has been described⁴¹⁻⁴⁸.

The upregulation of miRNAs let-7i-5p and miR-98-5p has been shown to decrease IL-13 levels which resulted in the resolution of airway inflammation⁵². In this study we identified both miRNAs to be increased in plasma upon higher TRAP levels, thus potentially indicating a protective mechanism against increasing inflammation in the airways and circulation²⁷. We also identified the asthma-associated miRNAs miR-223-3p with decreased and miR-574-5p with increased plasma levels upon higher TRAP exposure. In lung tissue of mice challenged with ovalbumin mimicking acute, intermediate and chronic asthma, the downregulation of miR-223-3p to short term, and the upregulation of miR-574 to long term exposure to allergens has been reported⁶².

Recently, TRAP exposure has also been linked to an increased risk of neurodegenerative diseases such as Alzheimer's and Parkinson's disease⁷. We observed significantly decreased plasma levels of the brain-enriched miR-433-3p (85 % of all subjects) after exposure. This

miRNA has also been found to be significantly decreased in the serum and cerebrospinal fluid of Parkinson's and Alzheimer's diseases patients when compared to healthy individuals⁶¹. The downregulation of cmiRNAs is unlikely to reflect cell damage as a consequence of toxic exposure, but rather suggests a biological response by a signalling molecule to a change in ambient air pollutant exposure intensity.

Plasma levels of another brain-enriched miRNA, miR-1224-5p, appeared increased in 80 % of the study participants. This miRNA has been experimentally shown to silence *LRRK2*, which is known to be down-regulated during pathogenesis of Parkinson's disease^{67,68}.

In patients diagnosed with Alzheimer's Disease, decreased serum levels of circulating miR-885 have been described⁵⁹. Also in our study we have observed decreased plasma levels of this miRNA in association with TRAP exposure. Furthermore, miR-107 and miR-34a-5p, both highly abundant in brain tissue, were found to be decreased in serum even in patients at the earliest stages of Alzheimer's Disease^{54,60}. While we observed increased miR-107 plasma levels with increased exposure intensity, miR-34a-5p levels were indeed decreased upon exposure.

Furthermore, we identified 4 TRAP-associated miRNAs, namely miR-25-3p, miR-148a-3p, miR-150-5 and miR-223-3p, which are known to be implicated in the pathogenesis and progression of kidney diseases⁵⁶. The miRNA which we identified most abundantly expressed in kidney, miR-148a-3p, was increased in plasma following higher TRAP levels. A closely related family member of this miRNA, miR-148b-3p, was found upregulated in peripheral blood mononuclear cells in patients suffering from IgA nephropathy⁶⁹. More recently, serum levels of miR-148b-3p have been described as a potential non-invasive biomarker for diagnosing IgA nephropathy⁷⁰. Interestingly, another study described low levels of miR-223-3p in circulating

endothelial cells as a noninvasive method for evaluating the severity of igA nephropathy⁷¹. In our analysis we found lower levels of miR-223-3p with increased TRAP levels. Our tissue specificity analysis revealed that miR-148a-3p and miR-223 are not enriched in kidney and were found abundantly expressed in other tissues (TSI < 0.37). However, we identified another 6 kidney-enriched miRNAs (TSI > 0.68) that have not been sufficiently described in literature and might play a role in environmentally induced kidney diseases.

We evaluated the potential biological function of the cmiRNA signature by determining target genes, pathways and disease associations. The analysis showed that relevant disease pathways such as microRNAs in cancer, breast cancer, non-small cell lung cancer and small cell lung cancer as well as signaling pathways such as the PI3K-Akt signaling pathway and the p53 signaling pathway are potentially perturbed by exposure to ambient air pollution.

An earlier study reported 2 vesicle-associated cmiRNAs to be increased upon short-term exposure to metal-rich PM (miR-128 and miR-302) in healthy workers¹³. In our analysis we observed decreased miR-128 levels but no change in miR-302 levels after short-term exposure. Another study on extracellular vesicle-bound cmiRNAs reported levels of 5 miRNAs to be changed after 6 months and 1 year in relation to ambient PM_{2.5} levels. Further 1 significantly decreased miRNA (miR-30d-5p) was reported after 1 day of exposure²⁵. This cmiRNA has also been identified in our analysis with significant decreased plasma levels already after 2 hours of exposure to TRAP.

In this study we applied NGS to capture the complete plasma circulating miRNome and therefore were able to detect cmiRNAs that have not been investigated in relation to TRAP exposure previously. Despite the relatively small sample size, the experimental cross-over design of the

study combined with the personal exposure monitoring enabled us to find significant associations of cmiRNAs and TRAP in the selected population.

The limited sample size, however, did not allow a meaningful analysis after stratification by disease state (COPD or IHD vs healthy) or gender. A hierarchical clustering analysis confirmed that cmiRNA expression is not confounded by disease status (data not shown). As a consequence of the study design in which COPD and IHD subjects were matched to healthy controls of the same age our analysis is limited to subjects of a mean age of 65.1 years (SD 7.7). Therefore the results need to be validated in other age groups to evaluate to which degree the results are transferable to other age groups. Due to the limited organ coverage of the human miRNA tissue atlas our analysis of miRNA tissue origin has been based on publically available sequencing data from various laboratories and therefore only suggests a potential origin of the detected cmiRNA. In summary, by applying stringent statistical analyses we have shown that the circulating miRNome is altered already after 2 hours of exposure to ambient air pollution in a dose- and pollutant species-dependent manner. The cmiRNA signature seems to be capable to identify molecular mechanisms that upon perturbation by TRAP exposure are possibly involved in the complex pathogenesis of multiple diseases. Strikingly, the miRNA profile identified already after 2 hours of TRAP exposure, presents specific associations with all major disease risks reported in relation to exposure to ambient air pollution. Next to the known disease risks that are affected by TRAP exposure our data also suggests aberrant abundance of circulating miRNAs that potentially have originated from the liver, pancreas and spleen. Since in addition, animal studies have demonstrated that in particular liver and spleen also present target organs for TRAP we suggest that future epidemiological studies should consider these organs to unravel their potential role in TRAP-associated pathology. Overall, our findings will not only contribute to the

understanding of the complex coherence of environmental exposure and miRNAs but also provide insights into the pathomolecular process of TRAP-induced diseases, and thereby substantiate to biomarker-based health risk assessment. Consequently, we propose that a cmiRNA signature comprised of organ-enriched miRNAs presents a highly specific candidate for biomarker-based health risk assessment allowing the early detection and prevention of TRAP-induced health outcomes.

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Author Contributions

J.K., T.M.K. and J.C.K. designed the research. K.F.C., P.Cu., P.Co., B.B., F.J.K. and P.V. organized the epidemiologic part of the work. J.K. and R.S. performed the experiments. J.K., F.C., K.V., M.C. and R.V. analyzed the data. T.M.K. and J.C.K. supervised the project. J.K., T.M.K. and J.C.K. co-wrote and all authors commented on the paper.

Additional Information

The authors declare no competing financial interests.